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Pathogenesis of ovine gamma herpesvirus 2 in rabbits involves productive infection of M-cells in the appendix

INAUGURAL-DISSERTATION

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Abstract

Sheep-associated malignant catarrhal fever (MCF), caused by the Ovine herpesvirus 2 (OvHV-2), is a sporadic and usually fatal infectious disease of cattle, other ruminant species, and swine. A system for propagation of OvHV-2 *in vitro* has not yet been identified and immunological tools for the identification of viral proteins are scarce. Therefore, the pathogenesis of OvHV-2 infection is poorly understood.

We have determined nucleotide sequences from cosmid-cloned viral DNA and identified two open reading frames, which were likely to encode for structural OvHV-2 proteins. ORF43 is related to UL6 of herpes simplex virus 1 (HSV-1), which encodes the pore forming capsid protein, required for packaging of viral DNA. ORF63, encoding a putative tegument protein, is related to UL37 of HSV-1 and HSV-2. GST-fusion proteins containing antigenic regions of these predicted proteins were generated and used to raise antisera for immunohistology.

Using these sera, productively OvHV-2-infected cells were detected in gut tissue of experimentally infected rabbits. They were identified as M-cells and adjacent epithelial cells in the appendix. Since M-cells interact closely with lymphocytes, which may harbor the OvHV-2 genome, we hypothesize that such interactions lead to local reactivation of OvHV-2 from latency, which then causes productive infection of epithelial cells in the vicinity. Thus, antisera raised against two predicted structural proteins of OvHV-2 were able to establish a link between cloned viral DNA and antigens present in tissues of diseased animals. Furthermore, the results reveal new aspects regarding the pathogenesis of MCF.

Introduction

Malignant catarrhal fever (MCF) is a sporadic and usually fatal infectious disease of cattle, other ruminant species, and swine (1,7,19,23,24,25). Major clinical symptoms include nasal and ocular discharge, conjunctivitis, gastroenteritis, hematuria, and fever (25).

There are two etiologically distinct forms of MCF: (i) a wildebeest-associated form, caused by alcelaphine herpesvirus 1 (AlHV-1), and (ii) a sheep-associated form (SA-MCF), occurring worldwide and implicated with Ovine herpesvirus 2 (OvHV-2). AlHV-1 was isolated a number of years ago and can be propagated in cell culture (6,28,31). Following this, the entire genomic sequence of AlHV-1 was determined relatively easily (13). Accordingly, AlHV-1 is a member of the genus Rhadinovirus within the subfamily *Gammaherpesvirinae*. In contrast, no system for propagation of OvHV-2 *in vitro* has yet been identified (7) and the sequencing of its genome is still incomplete. Its taxonomical status is not secure, although it has been designated as a gammaherpesvirus on the basis of DNA homology to AlHV-1 (5).

Both AlHV-1 and OvHV-2 are asymptomatic in their natural hosts, i.e. wildebeest and sheep (6,19). However, natural or experimental infection of other ungulates or swine as well as rabbits and hamsters may lead to a fatal lymphoproliferative disease during which viral DNA can be detected and quantified in white blood cells as well as in proliferating lymphocytes within affected organs (1,3,19,20,29,30,37). Yet, there is very little evidence of productive viral replication in the same cells (7,32). Thus, it appears as if OvHV-2 would initially favor the establishment of latency, not initiation of the lytic cycle. While this is not uncommon for gamma herpesviruses, these circumstances make it very difficult to develop specific immunological tools for the identification of lytically infected cells. Although there is a monoclonal antibody against AlHV-1, which binds to a shared epitope of AlHV-1 and

OvHV-2 (22), monospecific antisera or monoclonal antibodies against OvHV-2 have not yet been raised. Thus, the study of the contribution of replicating virus to the pathogenesis of OvHV-2 within the infected host has made but little progress.

To overcome these difficulties, a cosmid library was constructed from an OvHV-2 infected cattle cell line that contained mainly circular virus genomes (29,32) and viral DNA sequences were determined (J. Rosbottom, G. Jayawardane, J. Stewart, H. Reid, and M. Ackermann, manuscript in preparation). Two open reading frames (ORFs) were identified, which revealed sequence homology to structural proteins of other herpesviruses. The homolog to OvHV-2 ORF43 has been reported to encode a minor capsid protein in AlHV-1 (13) and corresponds to UL6 of herpes simplex virus 1 (HSV-1)(26). ORF63 matches to the gene for a phosphoprotein of AlHV-1, which is incorporated into the tegument and which is related to the gamma 1 gene UL37 of HSV-1 and HSV-2 (11,36,48). In order to generate antisera, glutathione S-transferase (GST) fusion proteins, containing antigenic parts of these proteins, were generated in *E. coli* and used for the immunization of rabbits and mice. The difficulty in the present setting was that a cell culture-adapted strain of OvHV-2 was not available for screening of the newly generated antisera. Therefore, the entire OvHV-2 ORFs were cloned independently into HSV-1-based amplicon vectors to be expressed in eukaryotic cells, either alone or as fusion proteins with enhanced green fluorescent protein (eGFP)(38).

HSV-1 amplicons are virus particles, which contain packaged foreign DNA in the place of the viral DNA (42). They are produced by cotransfecting the HSV-1 genome, deleted for the packaging signals, together with plasmid cloned DNA, containing the gene of interest as well as the HSV-1 origin of DNA replication and the HSV-1 packaging signal (34). These amplicon particles can then be used to transduce susceptible eukaryotic cells. Transduction leads to the expression of the foreign genes in these cells and the amounts of gene expression can be

controlled by using a promoter of choice (38,47). Successful gene expression can be monitored directly under the fluorescent microscope, provided that the genes of interest had been fused to sequences encoding for the eGFP. This approach represents, therefore, an alternative to the generation of stably transformed cell lines, which may or may not express the desired antigens (43). Pseudo-tissues can then be prepared from a mixture of transduced and non-tranduced cells and treated as if they were infected tissues obtained from animals. This allows setting of the conditions for the unknown antisera that are required for the detection of productively infected cells from animals.

Here we report on the production of antisera against the proteins encoded by ORF43 and ORF63 of OvHV-2. These sera were used for detection of OvHV-2 lytic antigens in experimentally infected rabbits (30). Productively OvHV-2 infected cells were detected in the appendix of such animals. These cells were further identified as epithelial cells and M-cells. Because M-cells play a role in the orchestration of the host's immune response, these findings may be relevant for the understanding of the pathogenesis of MCF in susceptible hosts. Furthermore, knowledge of these findings may be important for the study of the pathogenesis of other gamma herpesviruses.

Materials and methods

Viruses and cells

Cell-associated infectious OvHV-2 (32) was derived from T cells isolated from a cow that subsequently succumbed to SA-MCF in 1994 (BJ1035, from frozen stock) and from the first passage of the same isolate in rabbits.

The HSV-1-based helpervirus-free amplicon sytem, consisting of BAC-cloned HSV-1-DNA without packaging signal (pHSVΔpacΔpacIΔ27Δkn; kindly provided by Dr. C. Fraefel) and cloned amplicon DNA (42), was used for the eukaryotic expression of OvHV-2 ORF's as described previously (15,33,34).

Modified Vero 2-2 cells (kindly provided by Dr. R. Sandri-Goldin) (41), needed for packaging of amplicon particles, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin and incubated in 5% CO_2 at 37°C.

Cosmid cloning, sequencing, and sequence interpretation

The DNA sequences of ORF43 and ORF63 were obtained as part of a larger project to sequence the entire viral genome (J. Rosbottom, G. Jayawardane, J. Stewart, H. Reid, and M. Ackermann, manuscript in preparation). Briefly, a cosmid library containing the OvHV-2 genome was made in Supercos 1 (Stratagene, Amsterdam, The Netherlands) essentially as described previously (8) and using total cellular DNA from an OvHV-2-positive cell line (BJ1035; (32)). An initial OvHV-2-positive cosmid clone was isolated from this library using a probe derived from the sequence of part of the ORF75 gene (5). Other cosmid clones were then isolated by walking using probes derived from the cosmid ends. Cosmid-cloned OvHV-2 DNA was subcloned in an M13 shotgun library and subclones were sequenced with BIG-dye terminator version 1.0

(Applied Biosystems, Rotkreuz, Switzerland) and analyzed on a MegaBace 1000 sequencer (Amersham Pharmacia, Biotech, Dübendorf, Switzerland). Computer assembly was done with the DNAstar software version 5.05. The redundancy of sequencing was 7. Open reading frames were determined using the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and compared with other herpesvirus genes using the NCBI BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of ORF43 and ORF63 have been submitted to Genbank and assigned the accession numbers AY366191 and AY366192 respectively. Phylogenetic trees were built using MegAlign (DNAstar software 5.05) using the Clustal W algorithm with default settings (45).

PCR amplification

DNA sequences encoding for the ORF43 region H284-N435 and the ORF63 region N833-L944 were amplified from cosmid C33-63 using primers containing *Eco*RI sites for cloning of the products into the pGEX-6P-1 vector (Table 1).

The entire coding sequences of ORF43 and ORF63 were amplified from the same cosmid but using primers containing *Bam*HI (ORF43) and *Eco*RI sites (ORF63) for cloning into a HSV-1-based amplicon vector (pHSVPrPUC, kindly provided by Dr. Howard Federoff). The coding sequence without stop codon of the enhanced green fluorescence protein (eGFP) was amplified from the pGFP-N3 vector (Clontech, Basel, Switzerland), using primers to generate *Xba*I sites (Table 1).

For PCR, 5 µl template (0.4 µg / µl C33-63 or 1.5 µg / µl pGFP-N3) was mixed with 10 µM of each primer (2 µl), 3 µl *Pfu* buffer (Stratagene, Amsterdam, The Netherlands), 1 µl Pfu polymerase (2.5 U / µl) (Stratagene), 1 µl dNTP (10 mM,

Amersham Pharmacia, Biotech, Dübendorf, Switzerland), and 16 μl of sterile distilled water.

Following initial denaturation at 95°C for 1 min, 35 cycles were completed with denaturation at 95°C for 1 min, annealing for 1 min at the temperature specified for each product in Table 1, and extension at 74°C for the time specified in Table 1. All PCR products were analyzed by agarose gel electrophoresis and digested with the appropriate enzyme for cloning.

Table 1. Primers for amplification of OvHV-2 DNA and eGFP.

Oligonucleotide[a]	Sequence (5'-3')[b]	Annealing (°C)/ Extension (min.)	Product length
fragment 43F	GATATCGAATTCCACCTAGATGAGTGTAGT[c]	50 / 1	478 bp
fragment 43R	TAGAGAATTCCTAGTTAACCTGGTTGGAT[c]		
fragment 63F	CTCCGAATTCAATCCCCAAATTTTCCTTGC[c]	52 / 1	356 bp
fragment 63R	ACGCGAATTCTTACAGCTCCTGCGTAGGCT[c]		
43F	GTCGGATCCAGGATAAAGCTCTTAGGTATG[d]	60 / 2.5	1735 bp
43R	AGCGGATCCCGGGGCCCGCTACTCAGTCG[d]		
63F	CCTCTCTGGAATTCATGATGGAGAACAAGC[d]	55 / 4.5	2886 bp
63R	TGGAATTCGGCTGTGAGGCCATGGCGCTACG[d]		
GFP-F	GACGGTACCTCTAGACCGGGATCCATCGCCACC[e]	55 / 1	763 bp
GFP-R	GCGGCCTCTAGACTTGTACAGCTCGTCCAT[e]		

[a]F: forward primer, R: reverse primer

[b]Restriction sites underscored: [c]EcoRI, [d]BamHI, [e]XbaI

Construction and expression of GST-fusion proteins

The ampicillin resistant pGEX-6P-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden), designed for isopropyl β-D-thiogalactoside (IPTG) inducible expression of glutathione S-transferase (GST) fusion proteins in *E. coli*, was

used. A multiple cloning site (MCS) downstream of the GST-gene and the adjoining site for cleavage with human rhinovirus 3C protease allows insertion of DNA sequences encoding for the antigenic region of choice. The regions of choice were amplified by PCR as described above and inserted into the *Eco*RI locus of the MCS. These plasmids were maintained in *E. coli* DH5α.

E. coli BL21 (Amersham Pharmacia Biotech, Dübendorf, Switzerland) were used for expression of protein according to the protocols of the manufacturer. Briefly, 400 ml LB medium (Chemie Brunschwig AG, Basel, Switzerland) containing 100 µg/ml Ampicillin were inoculated at 37°C with 4 ml overnight culture. At OD_{550} = 0.5-0.7, protein expression was induced by adding IPTG (Qbiogene, Illkirch Cedex, France) to a final concentration of 1 mM and the temperature was reduced to 30°C. After 4 hours bacteria were harvested by centrifugation at 3000 x g, 4°C, 10 minutes in a GSA rotor. The cell pellet was suspended in 20 ml STE buffer (50 mM NaCl, 50 mM Tris base pH 8.0, 5 mM EDTA). Each 200 µl protease-inhibitor (Protease Inhibitor Cocktail, containing AEBSF, EDTA, Bestatin, Pepstatin A and E64, Sigma, Buchs, Switzerland) and 200 µl lysozyme (100 mg/ml) were added. After incubation on ice for 15 minutes 200 µl 1M DTT and 2 ml 10% sarcosyl were added and the sample sonicated for 1 minute. Debris was removed by centrifugation. 4 ml Triton X-100 (10%) and STE-buffer was added to a final volume of 40 ml. After incubation at room temperature for 30 minutes, 500 µl bed volume of prepared glutathione sepharose (Amersham Pharmacia Biotech, Dübendorf, Switzerland) in phosphate buffered saline (PBS-A, containing 0.14 M NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM Na_2HPO_4) was added and the sample incubated at 4°C for 4 hours. The beads were harvested by centrifugation at 1800 x g for 10 minutes. After 3 times washing with PBS-A, protein was eluted from the beads by the addition of 3 times 0.5 ml 10 mM glutathione elution buffer (Amersham Pharmacia Biotech, Dübendorf, Switzerland). The protein concentrations were determined in a GeneQuant II Spectrophotometer (Amersham Pharmacia

Biotech, Dübendorf, Switzerland) at 280 nm, based on the predicted molecular weights and calculated molar extinction coefficients (18). The eluates were stored at 4°C until further use.

Construction and packaging of amplicon vectors

The plasmid pHSVPrPUC (kindly provided by Dr. C. Fraefel) used as amplicon vector contains the HSV-1 immediate early (IE) 4/5 promoter, followed by an MCS for the insertion of a cassette containing the gene of interest, the origin of DNA replication (oriS) from HSV-1, and the HSV-1 packaging signal. Among other sites, the MCS containes an *Xba*I site upstream of the *Bam*HI and the *Eco*RI site. The OvHV-2 full length ORFs 43 and 63 were amplified by PCR as described above and cloned into the MCS of pHSVPrPUC, using the *Bam*HI locus for ORF43 and the *Eco*RI locus for ORF63 (Table 2). In addition, both ORFs were tagged to generate N-terminal eGFP-fusion proteins. For this, the eGFP gene was amplified by PCR and cloned into the *Xba*I site within the MCS of the recombinant vectors pHSVPrPUC-43 and pHSVPrPUC-63 in frame with either ORF43 or ORF63 (Table 2).

For packaging, 1.2 x 10^6 Vero 2-2 cells, seeded in Petri dishes (diameter of 6 cm), were cotransfected with 250 µl Optimem (Invitrogen, Basel, Switzerland) mixed with 0.5 µg Amplicon-plasmid (pHSVPrPUC-GFP-43 0.5 µg/µl, pHSVPrPUC-GFP-63 0.58 µg/µl), 0.2 µg pEBHICP27 (0.65 µg/µl) and 2.0 µg pHSVΔpacΔpacIΔ27Δkn (0.435 µg/µl). To this mixture 250 µl Optimem containing 16.8 µl Lipofectamin (Invitrogen, Basel, Switzerland) was added. After incubation at room temperature for 45 minutes 0.9 ml Optimem was added. Cells were then incubated in 5% CO_2 at 34°C for 5 hours. After 5 hours the cotransfection mixture was replaced by DMEM supplemented with 6% FCS. After 60 hours the cells were scraped from the dish using a rubber policeman.

To harvest amplicon particles, three cycles of freezing-heating were performed using dry ice / Ethanol and a 37°C water bath before sonication for 10 sec with 20% output. Cell debris was removed by centrifugation for 10 min at 1400 x g and the supernatant was stored at -80°C until further use.

Table 2. Properties of two putative structural proteins of OvHV-2 and of fusion proteins derived thereof.

	ORF43	ORF63
OvHV-2 protein		
Identity (similarity) to AlHV-1	83% (92%)	61% (77%)
Amino acids (aa)	562	947
Predicted MW (kDa)	63.7	106.5
Predicted localization	Capsid	Tegument
Fusion protein		
Construct	N-GST-3C-43fragment-C	N-GST-3C-63fragment-C
OvHV-2-fragment (aa)	H284-N434 (151)	N833-L944 (111)
Amino acids (fragments)	385 (234+151)	345 (234+111)
Predicted MW	44.3 (27.2+17.1)	39.3 (27.2+12.1)
Observed mobility (Mr)	50 (30+25)	43 (30+19)
Amplicon-mediated		
Construct	N-(eGFP)-ORF43-C	N-(eGFP)-ORF63-C

Immunization of rabbits and mice

New Zealand White rabbits and C57BL/6 mice were immunized with purified fusion proteins. Preimmune sera were collected from all animals prior to the first vaccination. Purified fusion proteins were dialyzed against phosphate buffered saline (PBS) and mixed (1:1) with adjuvant before being applied. Freund's complete adjuvant (FCA) was used for the first immunization; Freund's incomplete adjuvant (FIA) for booster injections. Rabbits were immunized with

100 µg protein in a volume of 2 ml, mice with 20 µg protein in a volume of 100 µl per injection. Rabbits received booster injections at monthly intervals, simultaneously with collection of serum samples. Mice received two immunizations at three week intervals. Mouse sera were collected two weeks after the second immunization and stored at -20°C until further use.

Antisera, conjugates, and substrates

In addition to the antisera described above, the following components were used: Mouse-anti-Vimentin (DAKO, Zug, Switzerland; code N1583); goat-anti-rabbit-horseradish peroxidase (HRP) conjugate (Bioreba, Reinach, Switzerland); anti-mouse-Envision (DAKO Envision™ System, horseradish peroxidase, Mouse; code K4001); DAKO Envision™ Doublestain System (code K1395); phosphatase conjugated anti-mouse-Envision (DAKO); Substrates: Aminoethyl Carbazole Substrate Kit (AEC-Kit, Stehelin AG, Basel, Switzerland). Fast red phosphatase chromogen solution (DAKO); goat-anti-rabbit serum conjugated with Cy™3 (Stehelin AG, Basel, Switzerland); chloronaphthol substrate solution with peroxide (4-chloro-1-naphtol tablets, Sigma, Buchs, Switzerland).

Protease digestion, SDS-Polyacrylamide gel electrophoresis (PAGE), and immunoblotting

To characterize the fusion proteins, GST was cleaved from the viral antigenic regions by digestion with human rhinovirus 3C protease (PreScission®, Amersham Pharmacia Biotech, Dübendorf, Switzerland). Loaded glutathione sepharose beads were washed with 10 x bed volume cleavage buffer containing 50 mM Tris HCl pH 7.0, 150 mM NaCL, 1 mM EDTA, 1 mM DTT at 5°C before PreScission protease (80 units/ml) was added and incubated over night at

5°C. The beads with bound GST were then removed by centrifugation at 1800 x g for 10 minutes.

Samples from different purification steps were mixed with SDS-loading buffer (62.5 mM Tris-HCl, pH 6.8, containing 25% glycerol, 2% sodium dodecyl sulfate, 0.01% bromphenol blue, 5% 2-mercaptoethanol; Bio-Rad Laboratories, AG, Reinach, Switzerland) and boiled for 10 min before being separated by SDS-PAGE containing 15% polyacrylamide and bisacrylamide (29:1) for crosslinking. After electrophoresis in Tris buffered (25 mM, pH 8.4) glycin (0.2 M) and SDS (3.5 mM), the proteins were transferred electrically to nylon sheets (Hybond-P, Amersham Pharmacia).

Immunoblotting was done at room temperature. The nitrocellulose membranes were blocked for 1 hour in PBS-A with 10% skim milk. Then, they were incubated for one hour with the rabbit sera of interest (each 1:1000 in PBS-A). After washing with PBS-A the blots were incubated for one hour with goat-anti-rabbit-HRP conjugate (1:1000 in PBS-A). Finally, the reaction was visualized using a chloronaphthol substrate solution.

Production and processing of pseudotissues

Pseudotissues were produced using the Thermo Shandon Cytoblock® Cell Preparation System (DAKO, Zug, Switzerland). Briefly, monolayers of Vero 2-2 cells in Petri dishes (diameter of 6 cm) were transduced with packaged amplicon particles at an m.o.i. (multiplicity of infection) of 0.1. Transduction efficiency was monitored after 48 hours, either directly by fluorescence of eGFP or by immunofluorescence as described below.

Cells were harvested by scraping into the supernatant with a rubber policeman, followed by centrifugation for 10 minutes at 1800 x g. The pellet was washed with PBS-A before being fixed for 1 hour at room temperature in phosphate

buffered (46 mM Na_2HPO_4 and 29 mM NaH_2PO_4, pH 7.0) 4 % formaldehyde. The pellet was then washed with 1% bovine serum albumine (BSA) solution in PBS before it was transferred to the Cytoblock Cell Preparation System and paraffin-embedded. The pellet was sectioned into 2 µm thin slices using a microtome (HM3555, Microm, Volketswil, Switzerland). The sections were mounted onto positive charged slides (SuperFrost Color, Menzel, Braunschweig, Germany) before being used in immunohistochemistry as described below.

Infection of rabbits and harvesting of tissues

Two New Zealand white rabbits were infected intravenously with cell-associated OvHV-2. One rabbit (MP02/1092) received 10^8 infected cattle T cells (BJ1035). The second rabbit (MP02/1445) received 10^8 infected rabbit T cells taken from BJ1035 passage 1. Rabbits were euthanized on day 16 (MP02/1445) and day 30 (MP02/1092), when rectal temperatures had risen to >40°C for two days. Various tissues that are affected by OvHV-2 MCF were collected (appendix, lung, spleen, liver, mesenteric lymph nodes and kidney) and a portion of each tissue fixed in either 10% formal saline or zinc fixative (zinc salts consisting of 0.1M tris base, 0.05% Ca acetate, 0.5% Zinc chloride and 0.5% Zinc acetate). The same tissues were taken from an uninfected control rabbit. Sections of rabbit tissue were mounted to slides as described above for pseudotissues.

Immunofluorescence

Incubations and washings were done at room temperature. Monolayers of transduced cells were fixed with methanol for 30 minutes before being incubated with the antisera of interest (diluted 1:1000 in PBS-A) for 1 hour. After washing with PBS-A, the Cy3-conjugate was overlaid for 1 hour. After further washing,

the cells were immediately observed using the fluorescent microscope. The FITC-filter (optimized for maximum emission at 507 nm) was used for the detection of eGFP-specific fluorescence, the DS-red filter (optimized for maximum emission at 583 nm) for Cy3.

Immunohistology

For immunohistochemistry slides, containing either rabbit tissue sections or pseudotissue sections, were deparaffinized on descending alcohol series and counter-stained with haemalaun for 3 minutes. All incubations and washings were done at room temperature. Endogenous peroxidase was blocked with water supplemented with 3% H_2O_2 and 0.2% NaN_3 for 10 minutes. To minimize unspecific reactions, the slides were incubated for 10 minutes with DAKO® Protein Block Serum Free Solution (code X0909). The slides were washed with PBS before a pronase-treatment (code S2013, DAKO®) for 5 minutes was performed.

After washing with PBS, the slides were incubated over night with 100 µl of primary antiserum at a dilution of 1:500. The next day the slides were washed and incubated for 30 minutes with the peroxidase conjugate, anti-mouse-Envision. After further washing, the slides were developed for 10-15 minutes with amino-ethyl carbazole (AEC) substrate (Stehelin AG, Basel, Switzerland).

The DAKO Envision™ Doublestain System (code K1395) was used to co-detect viral antigens (ORF 43 or 63 proteins) and vimentin with the following modification: to avoid unspecific staining of rabbit tissues with anti rabbit-labelled polymers, an anti mouse polymer-horseradish peroxidase-conjugated antibody was used (code K4001, DAKO Envision™). The slides were treated as described above. After development of the anti-viral staining mouse-anti 43 or mouse-anti 63 with Diaminobenzidine (DAB) from the doublestain kit for 5 to

10 minutes, the slides were further incubated with DAKO® Double Staining block solution and DAKO® Protein Block Serum Free. Then, mouse-anti-vimentin was added for 30 minutes. The secondary staining was developed by the alkaline phosphatase conjugated anti-mouse / anti-rabbit reagent from the doublestain kit, followed by visualization using the fast red chromogen solution for 5 to 10 minutes.

Results

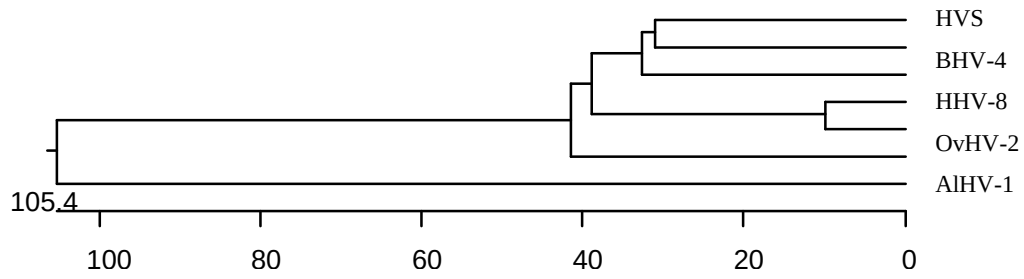
Identification of open reading frames encoding for potential OvHV-2 structural proteins

Nucleotide sequences from cosmid-cloned OvHV-2 DNA were compared with the DNA-sequence of Alcelaphine herpesvirus 1 (13) in order to identify putative structural proteins of OvHV-2. Among others, these analyses (summarized in Table 2) revealed two open reading frames (ORFs) whose predicted amino acid (aa) sequences were similar to those of a minor capsid protein (ORF43) and a tegument protein (ORF63), respectively, from AlHV-1.

With 82% identity on the amino acid sequence level, the predicted OvHV-2 ORF43 product was most closely related to its homolog in AlHV-1. Still 48% identity was found relative to the homologs in other gamma herpesviruses, i.e, BHV-4 and HHV-8. A marginal similarity of 22% was present even with the corresponding protein of the Bovine alpha herpesvirus 1 (BHV-1). The similarity of the ORF63 product to the homologs in other gamma herpesviruses ranged from 23% (HHV-8) to 18% (EBV) but the closest relation was again found with AlHV-1 (61.6% identity). Phylogenetic trees (Figure 1) showing the relations of these proteins with the corresponding proteins from other herpesviruses were calculated on the basis of the Clustal W algorithm for multiple alignment.

Overall, the results concerning these two OvHV-2 proteins confirmed that OvHV-2 should be classified among the gamma herpesviruses. Furthermore, it may be expected that antisera against these two OvHV-2 proteins should be able to detect OvHV-2 in productively infected cells.

A



B

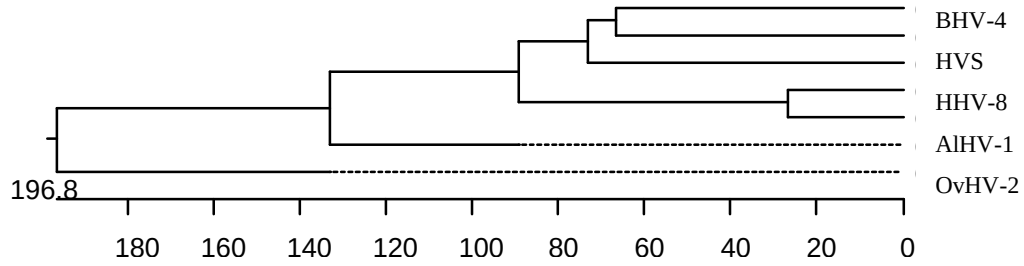


Figure 1. Dendrograms depicting the relationship between selected herpesviruses. The data are based on amino acid sequences of (A) ORF43 homologues and (B) ORF63 homologues. Branch lengths are proportional to the distance between the respective sequences. The dendrogram was obtained using Clustal alignment from MegAlign (DNASTAR Programs package).

Construction and expression of GST-fusion proteins containing OvHV-2-specific fragments

In order to generate antisera for the identification of these putative structural viral proteins, antigenic parts of the ORF43 and ORF63 encoded proteins were expressed as GST-fusion proteins (Table 2), purified as described in Materials and methods, and used for the immunization of rabbits and mice.

The specificity of the resulting sera was tested on Western immunoblots (Figure 2) using cleaved and uncleaved fusion proteins as antigens. The results indicated that both anti-ORF43 and anti-ORF63 sera recognized the GST-fragment in both of the uncleaved fusion proteins as well as the GST of a control preparation without viral antigenic region. In contrast, the band representing the cleaved

ORF43-region was stained only by the anti-ORF43 but not the anti-ORF63 serum. Similarly, the cleaved ORF63-region was immune stained exclusively by the corresponding immune serum. These results indicated that both the rabbit sera (Figure 2) and the mouse sera (data not shown) specifically recognized the antigen that had been used for immunization.

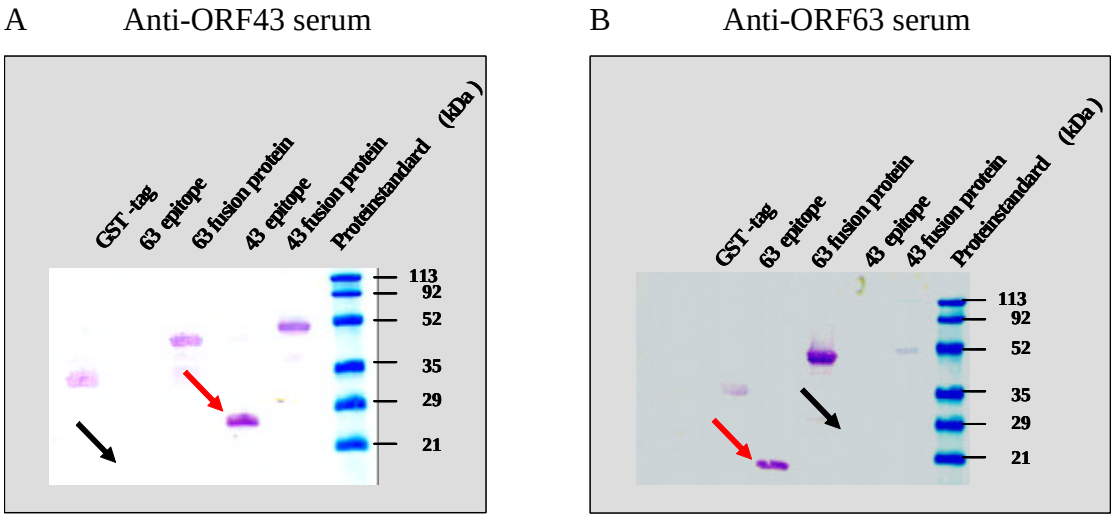


Figure 2. Characterization of fusion proteins and immune sera by Western immunoblots. The following samples were separated by SDS PAGE and blotted on nitrocellulose membranes: GST-tag alone (lanes 1), cleaved ORF63 fragment (lanes 2), ORF63 fusion protein (lanes 3), cleaved ORF43 fragment (lanes 4), ORF63 fusion protein (lanes 5), molecular weight marker (lanes 6). (A) immune-stained with anti-ORF43 serum, (B) with anti-ORF63 serum. Red arrows point to the specifically stained bands, representing the cleaved viral antigenic regions. Black arrows point to the location where the unrelated antigen is expected to migrate.

Amplicon-mediated expression of full length ORF43 and ORF63

Since productively OvHV-2 infected cell cultures were not available, ORF43 and ORF63 were heterologously expressed in eukaryotic cells. The entire ORFs were cloned into HSV-1-based amplicon vectors, either alone or as fusion proteins with eGFP (Table 2). The amplicon vectors were packaged into HSV-1

particles, which then were used to transduce Vero 2-2 cells. Successfully transduced cells were identified by the expression of eGFP. Representative results are shown in Figure 3. Green fluorescence in the cytoplasm of cells was observed starting from 6 hours post transduction. At 48 hours, the time of maximal fluorescence, the cells were fixed and incubated with the rabbit immune sera followed by a Cy3 labelled conjugate. While the sera did not react with cells transduced with the unrelated construct, red fluorescence was observed in cells transduced with either one of the ORF43 amplicons and immune stained with the anti-ORF43 serum. Similarly, eukaryotically produced ORF63 protein was detected by the anti-ORF63 antiserum. Green and red fluorescence co-localized in cells transduced with the fusion proteins and stained with the corresponding antiserum. No differences in the immune reactivity of either fusionproteins or polypeptides without eGFP-tag were observed (not shown).

These results indicated that the amplicon-mediated expression of OvHV-2 ORF43 and ORF63 proteins was successful and that, in the absence of other viral proteins, these polypeptides accumulated in the cytoplasm of the transduced cells. The antisera, raised against prokaryotically expressed fragments of the same proteins, were able to recognize their targets in the native environment of the full-length proteins.

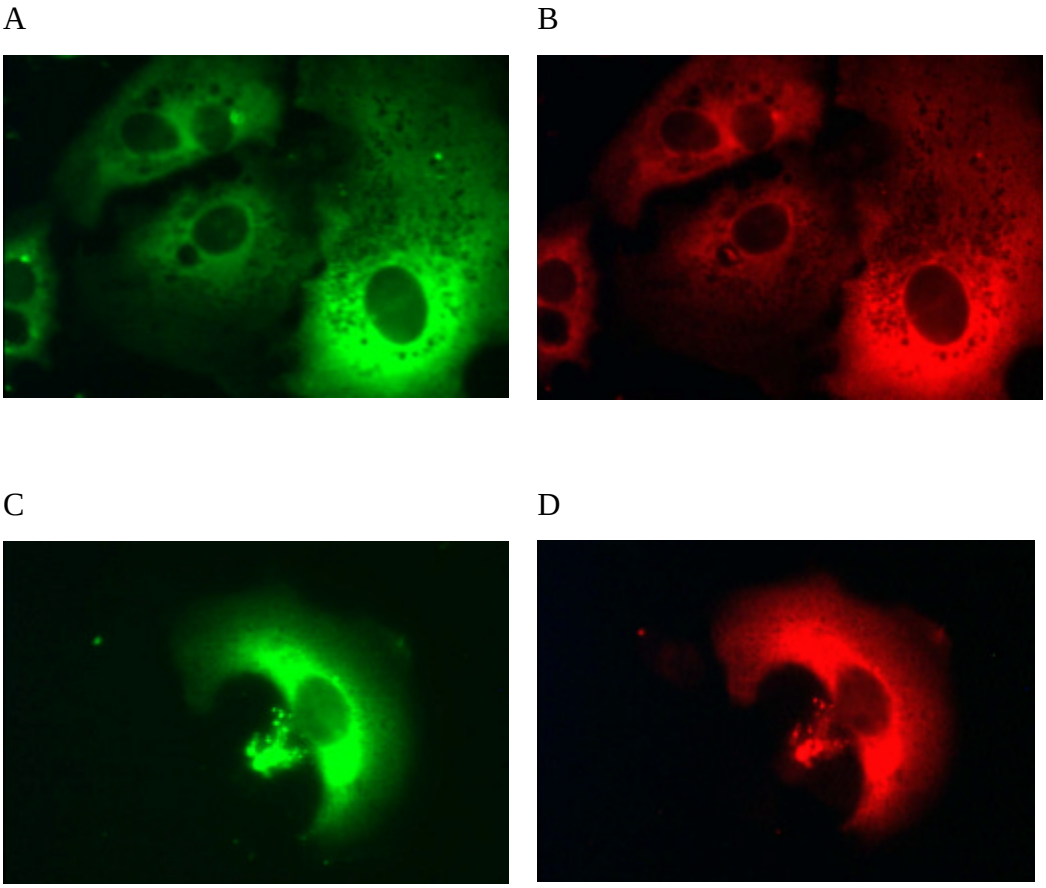


Figure 3. Eukaryotic expression of ORF43 and ORF63. VERO 2-2 cells transduced with an amplicon encoding for the eGFP-ORF43 (A+B) or eGFP-ORF63 (C+D) fusion proteins. Cells were observed under the fluorescence microscope using filters specific for eGFP (A+C) or Cy3 (B+D).

To adapt immunohistological detection of OvHV-2 antigens to the use of formaldehyde-fixed and paraffin-embedded tissue, artificial tissues were prepared from a mixture of untransduced and transduced cells. For this purpose, monolayers of Vero 2-2 cells were transduced at a ratio of 10% with amplicons expressing the eGFP-fusion proteins. Green fluorescence served as indicator for the transduction ratio. 48 hours post transduction, the cells were trypsinized, pelleted, and placed into pre-formed gelatin-pockets to form a tissue-like body as described previously (9). Cell pellets were fixed with formalin and embedded in paraffin to simulate possible fixation of tissue samples. These pseudo-tissues were then sectioned and mounted. Between two and three cells per 10^4 retained fluorescent activity throughout this treatment. Thereafter, the slides were

incubated with antisera against ORF43 or ORF63 and conjugates as described in Materials and methods. Approximately the same number of cells that had retained fluorescent activity also reacted positively by immunohistology (Figure 4). Apparently, a major amount of the proteins detected by immunohistology was present in the cytoplasm of the transduced cells.

These results indicated that a loss in sensitivity amounting to a factor of 1'000 had to be attributed to the procedures of fixation and embedding. Thus, the produced antisera could be expected to retain their ability to recognize their antigens, even in formalin-fixed and paraffin-embedded tissue.

A B

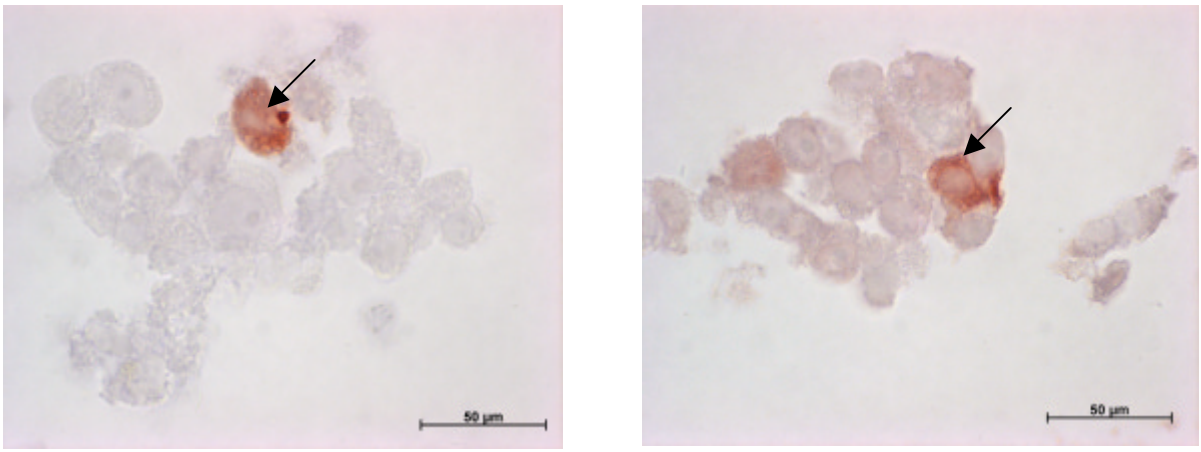


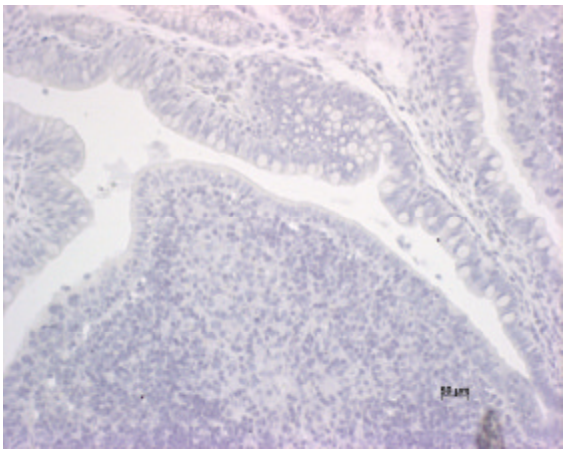
Figure 4. Detection of eukaryotically produced ORF43 and ORF63 proteins in formaldehyde-fixed pseudotissues. Cells were transduced with an amplicon encoding for ORF43 (A) or ORF63 (B) and stained with anti-ORF43 serum or anti-ORF63 serum, respectively. Arrows point to positive cells.

Detection of OvHV-2 protein in tissue of infected rabbits

Recently, Rosbottom and others (2002) reported that rabbit T cell lines supported productive OvHV-2 replication. In addition, infectious material had been harvested from gut-associated lymphoid tissue such as mesenteric lymph nodes and appendix (37). It was hypothesized that structural viral proteins might be detected in such tissues of infected rabbits. When sections from tissues (see

Materials and methods) of OvHV-2-infected or non-infected rabbits were subjected to immunohistology with mouse antisera against the putative tegument protein 63, a specific staining was detected exclusively in sections from the appendix of OvHV-2-infected animals. A strong signal was visible in the basal portion of the top layer of the dome epithelium of infected (Figure 5B) but not uninfected rabbits (Figure 5A). The signal was absent when preimmune sera were used (Figure 5D). This staining pattern and the location of positive cells suggested that cells positive for OvHV-2 tegument protein 63 might be M-cells. Since vimentin is considered as a marker for rabbit M-cells (16,17), the same tissues from infected or uninfected rabbits were subjected to a consecutive staining with a monoclonal antibody against vimentin. While, tegument protein 63 was not detected in tissue from uninfected rabbits, M-cells were stained in the same section (Figure 5C), essentially at the location, where viral protein had been observed in infected rabbits. In contrast, double positive staining was detected in appendices from infected rabbits, mostly at the periphery of the domes (Figure 5E,F). Thus, M-cells were identified as harboring structural protein of OvHV-2. Anti ORF43 antisera gave very similar staining patterns (not shown).

A



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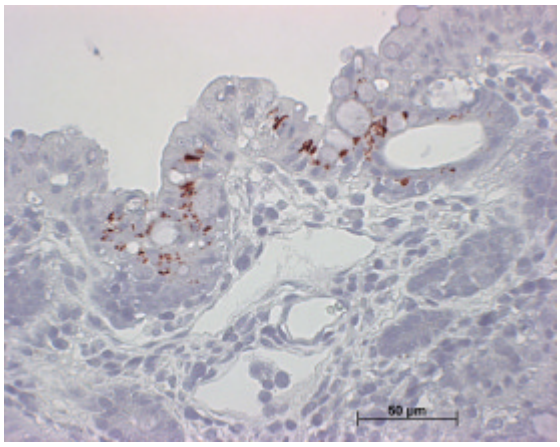

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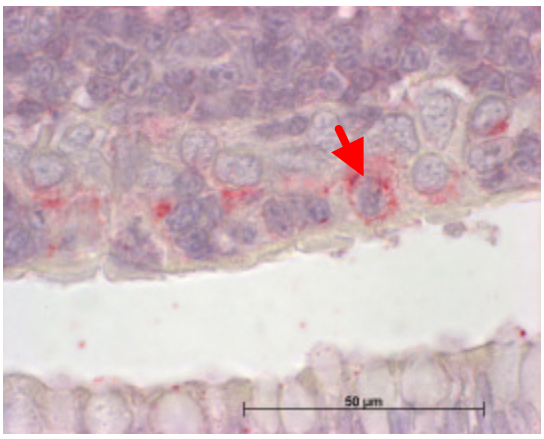

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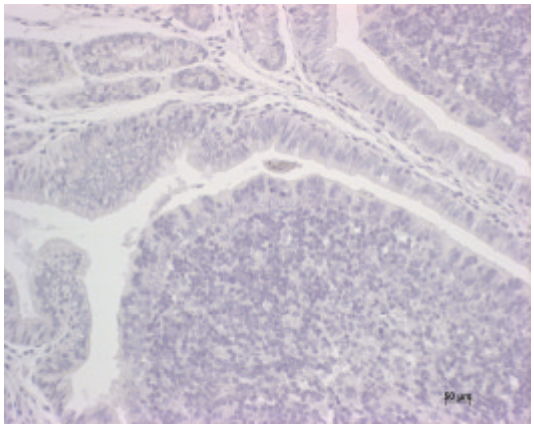

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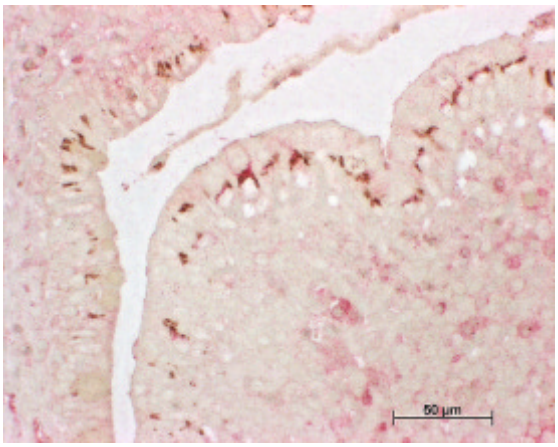

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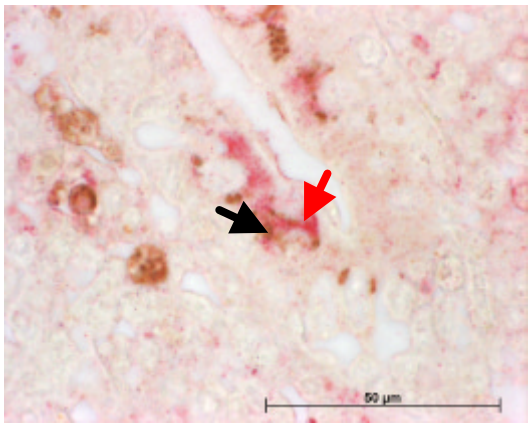

Figure 5. Detection of ORF63 protein in M-cells. Histological sections from the appendix of OvHV-2 infected (B,D,E,F) or uninfected (A,C) rabbits were subjected to immunohistology. Sections were stained with antiserum against tegument protein 63 (A+B) or double stained with the ORF63 serum and a monoclonal antibody against vimentin (C,E,F). (D) Section stained with pre-immune serum. Black arrow: ORF63 protein; red arrow: vimentin. Bars: 50 µm.

A second species of positive cells was identified as epithelial cells of the appendix. Figure 6 (A) shows a positive section of tissue from an OvHV-2-infected rabbit labelled with anti-ORF43 serum. Anti-ORF63 serum gave very similar staining patterns (not shown). In contrast, tissues from uninfected rabbits revealed no immunostaining (Figure 6B).

These results suggested that productive OvHV-2 infection in rabbits was ongoing in epithelial cells as well as in M-cells of the appendix. Simultaneously, the final link could be established between cosmid cloned OvHV-2 nucleotide sequences, antigens and antisera produced on this basis, and viral antigens *in vivo*.

A B

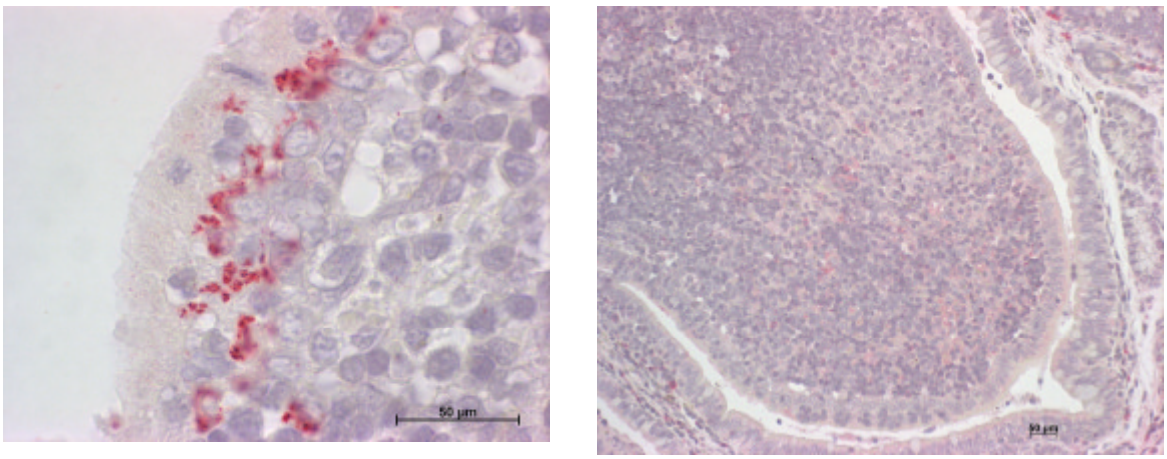

Figure 6. Detection of ORF43 protein in epithelial cells from OvHV-2-infected rabbits. Rabbits were inoculated with OvHV-2 and sacrificed upon appearance of MCF-like symptoms. Gut tissues from the appendix were treated for immunohistology with anti-ORF43 serum as described in Materials and methods. (A) Positive epithelial cells with perinuclear cytoplasmic antigen. (B) Noninfected rabbit.

Discussion

The main obstacle for better understanding the pathogenesis of sheep-associated malignant catarrhal fever is based on the lack of tools to study and trace the replication and biological properties of its causative agent, the ovine gamma herpesvirus type 2. Production and characterization of specific antibodies against this virus is still difficult because OvHV-2 cannot serially be propagated in cell cultures.

To overcome these shortcomings, we took advantage of genomic nucleotide sequences, made available after cloning of the viral DNA into a cosmid library. Among the sequences of the first cosmid analyzed, two genes emerged encoding for potential structural proteins of OvHV-2, i.e. ORF43 and ORF63. Comparison of these ORFs with corresponding genes of other herpesviruses confirmed that OvHV-2 should be classified among the gamma herpesviruses and that AlHV-1 is a distinct but close relative (5).

Fragments of these ORFs containing predicted antigenic portions of the encoded proteins were fused to the GST-gene, expressed in *E. coli*, and used to immunize rabbits and mice for the generation of specific antisera against these putative OvHV-2 structural proteins. Comparison of the amino acid sequence of the selected antigenic region of ORF43 with AlHV-1 revealed a high degree of similarity. Although not tested, the generation of potentially cross reacting antibodies in the immune sera can, therefore, not be excluded. In contrast, the antigenic region of ORF 63 shares only one stretch of 12 aa with the corresponding sequence of AlHV-1 ORF63. Therefore, crossreaction of these antisera with AlHV-1 is not likely.

Our approach for generating antisera represents a standard procedure, which is often used to identify thus far unknown viral proteins (2,40). However, the difficulty in the present setting was that a cell culture-adapted strain of OvHV-2 is not available for screening of the newly generated antisera. Therefore, the entire OvHV-2 ORFs were cloned independently into Herpes simplex type 1-

based amplicon vectors to be expressed in eukaryotic cells, either alone or as fusion proteins with enhanced green fluorescent protein (eGFP)(38). These vectors were packaged into HSV-1 particles, which then were used to transduce Vero cells in order to eukaryotically express the OvHV-2 genes and use the transduced cells as a target for the newly raised antisera. Fluorescence from the eGFP-tags was used for controlling the transduction efficiency. Mixtures of transduced and non-transduced cells were used to form pseudo-organs, which could be sectioned in either a native or a fixed state, similar to conventional histopathological examinations. This approach represented an alternative to the generation of stably transformed cell lines, which express the desired antigens. It is well known that such cell lines sometimes are difficult to establish (43). Even if this task is done successfully, the problem of stable gene expression remains, not to speak of the production of sufficient amounts of the desired proteins within those cells. Since amplicon stocks can be titrated and used in a reproducible fashion, rendering the desired multiplicities of transduction, they bear also clear advantages over transient expression methods in eukaryotic cells by transfection or lipofection. Using these pseudo-organs in immunohistological experiments, it was possible to characterize the newly raised antisera with the aim to detect OvHV-2-antigen in tissues from infected animals.

Finally, by means of immunohistology, tissues from infected and non-infected rabbits allowed to establish the link between replicating OvHV-2, i.e. productively infected cells, and cloned DNA sequences. Tissues found to contain OvHV-2-antigens by immunohistology revealed also the corresponding mRNAs as analyzed by Taqman-RT-PCR (data not shown). Moreover, M-cells as well as epithelial crypt cells in the appendix of the rabbits were identified as being productively infected with OvHV-2.

Typically, only a subset of cells supports lytic replication by gamma herpesviruses. Tissues and blood cells obtained from diseased individuals rather

carry virus in various stages of latency (10,12,14,44). Therefore, only a minority of cells in the infected organism can be expected to carry antigen consisting of structural viral protein. However, accumulation of viral structural proteins can be expected in tissues predisposed for virus excretion. It was therefore not surprising that the majority of rabbit tissues reacted negatively with the newly developed antisera. Furthermore, a number of sheep tissues, previously tested positive for OvHV-2 DNA by quantitative fluorogenic PCR (20), did not react with these antisera (data not shown). Since infectious material had been harvested from gut-associated lymphoid tissue of OvHV-2-infected rabbits (39), mesenteric lymph nodes and appendices collected from such rabbits were likely to contain a certain number of lytically infected cells. Furthermore, the characteristic features of the predicted proteins chosen for detection makes it likely that they may be used as markers for lytic infection.

According to its predicted amino acid sequence, the ORF43 protein is a homolog of herpes simplex virus type 1 (HSV-1) UL6. Its gene product is supposed to form the portal for entry of the viral DNA into preformed capsids (26). Therefore, detection of the ORF43 protein in rabbit M-cells and epithelial cells of the appendix, is indicative for the replicative state of OvHV-2 in these cells.

The staining patterns found with the ORF63 proteins in our experiments resemble very much to the ones described for its homolog in HSV-2, the UL37 protein. In HSV-2-infected cells, the UL37 protein accumulates as a punctate structure in the perinuclear region, whereas it is mainly found in the cytoplasm of transfected cells without ongoing viral replication (48). Considering that the ORF63 protein of OvHV-2 may behave in a similar way, its punctate to diffuse intracytoplasmic pattern in rabbit M-cells argues for ongoing replication of OvHV-2. In contrast, the UL37 protein of HSV-1 is diffusely distributed in the cell but more abundant in the cytoplasm than in the nucleus and constitutes a minor tegument protein (11). Yet, the UL37 product of HSV-1 is synthesized

late in infection (gamma 1 protein), which also supports its usefulness as a marker for lytic replication (36).

M-cells are specialized epithelial cells, located in the lymphoid tissue of the gut, including Peyer's patches, and capable of antigen presentation (4,21,27,35,39,46). They are thought to take up antigens from the intestinal lumen in order to present them directly to lymphocytes within the patch. Interestingly, antigens that enter M-cells are likely to not be degraded but rather passed on to other cells or to the intercellular space. Indeed, soluble macromolecules, small particles, and even whole organisms are transported by M-cells. Therefore, some pathogens, such as salmonellae, Yersinia, Listeria, and reoviruses can use M-cells as a portal to enter the body. Since intravenous inoculation of OvHV-2 had been used for infection of rabbits, it is unlikely that this pathway actually played a role under our experimental conditions. Yet, M-cells interact closely with lymphocytes, which may harbor the OvHV-2 genome. Periods of interaction between the two cell types may then lead to reactivation of OvHV-2 from latency, which would eventually result in the synthesis and accumulation of structural viral proteins in M-cells. Under these conditions, it seems to be logical that we were able to detect structural OvHV-2 antigens in M-cells as well as in epithelial cell in their vicinity.

Moreover, since antigen presenting cells play an important role in the initiation and quality of the adaptive immune response, the infection of M-cells may bring some dramatic consequences with it. For example OvHV-2 encodes for an IL-10 homolog (J. Rosbottom, G. Jayawardane, J. Stewart, H. Reid, and M. Ackermann, manuscript in preparation), which might be able to deviate the developing immune response in a fashion that is specific for the infected host species. Since sheep but not cattle or swine underwent co-evolution with OvHV-2, this may ultimately explain why the pathogenesis of the OvHV-2 infection proceeds differently in various animal species.

In conclusion, antisera raised against two predicted structural proteins of OvHV-2 were able to establish a link between cloned viral DNA and antigens present in tissues of diseased animals. Since these tissues also contain infectivity, it is most likely that these antisera actually recognize lytically infected cells. The data gained in this study shed light on new aspects to understanding the pathogenesis of MCF in various animal species.

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